FEBS Letters 579 (2005) 4430–4436 FEBS 29798

Inhibitor-assisted refolding of protease: A protease inhibitor as an intramolecular chaperone

Shuichi Kojima*, Akane Iwahara, Hideyuki Yanai

Institute for Biomolecular Science, Gakushuin University, Mejiro, Tokyo 171-8588, Japan

Received 29 March 2005; revised 9 June 2005; accepted 28 June 2005

Available online 20 July 2005

Edited by Felix Wieland

Abstract *Pleurotus ostrearus* proteinase A inhibitor 1 (POIA1), which was discovered as a protease inhibitor, is unique in that it shows sequence homology to the propeptide of subtilisin, which functions as an intramolecular of a cognate protease. In this study, we demonstrate that POIA1 can function as an intramolecular chaperone of subtilisin by in vitro and in vivo experiments. The specific cleavage between POIA1 and the mature region of subtilisin BPN′ occurred in a refolding process of a chimera protein, and *Bacillus* cells transformed with a chimera gene formed a halo on a skim milk plate. The mutational analyses of POIA1 in the chimera protein suggested that the tertiary structure of POIA1 is required for such a function, and that an increase in its ability to bind to subtilisin BPN′ makes POIA1 a more effective intramolecular chaperone.

Keywords: Intramolecular chaperone; Protease; Protease inhibitor; Propeptide; Subtilisin

1. Introduction

The three-dimensional structures of proteins are generally determined by their primary structures and therefore basically refold spontaneously from a denatured state. However, there are many proteins that require molecular chaperones for the correct folding of their tertiary and quaternary structures. The propeptides of proteases are unique among various molecular chaperones, because they are degraded by a cognate protease after the single-turnover catalysis of protease folding [1–3]. Many propeptides are located between a signal peptide and the mature regions of the precursor forms of proteases, and therefore named "intramolecular chaperones" [1]. The propeptides of subtilisins have been most extensively studied. Although they lack three-dimensional structures in isolated form, they acquire a tertiary structure by forming a complex with their cognate protease [4–6]. They also function as protease inhibitors by binding to the substrate-binding pocket of proteases using their C-terminal region [7–9]. Attempts to introduce the tertiary structure into the isolated form of the propeptide of subtilisin BPN′ by amino acid substitutions by Bryan's [10,11,13,14] and our groups [12] have been successful. Moreover, the close relationships between tertiary structure formation and increases in activities as a molecular chaperone and a protease inhibitor have been reported [10–15].

Pleurotus ostreatus proteinase A inhibitor 1 (POIA1) and yeast proteinase B inhibitor 2 are proteins that were isolated as protease inhibitors composed of about 70 residues excluding Cys residues [16,17] and showing amino acid sequence homologies of approximately 30% to the propeptides of subtilisins. Our mutational analyses suggested that they are unique protease inhibitors that utilize their C-terminal regions as reactive sites [18,19]. Although their wild-type proteins function as digestible temporary inhibitors when subtilisin BPN′ was used as a target protease, mutated proteins whose C-terminal six residues had been replaced by those of the propeptide of subtilisin BPN′ were converted into stronger and resistant inhibitors. In the C-terminal region, the C-terminal residue was considered the most important in binding to the P1 pocket of subtilisin BPN′. Recently, we have determined the three-dimensional structure of wild-type POIA1 by NMR, and found that it is structurally very similar to the propeptide of subtilisin BPN in the complex with subtilisin BPN [20]. On the basis of these findings, in this study, we determined whether POIA1 can function as an intramolecular chaperone by in vitro and in vivo experiments. Through this study, we may shed light into the molecular mechanisms of propeptide-mediated refolding of subtilisin from different viewpoints.

2. Materials and methods

2.1. Construction of expression plasmid in Escherichia coli of pro-subtilisin BPN′ or chimera protein of POIA1 and subtilisin BPN′ with Ser221 → Cys substitution

The propeptide-encoding region of the wild-type subtilisin BPN′ gene was initially substituted with a mutated POIA1 (POIA1cm) gene in which the C-terminal six residues had been substituted with those of the propeptide of subtilisin BPN′. The plasmid pSUB-N encoding the N-terminal half of prepro-subtilisin BPN′ [21] was digested with *Eco*RI and *Spl*I to remove the promoter- and propeptide-encoding regions of subtilisin BPN′. A truncated POIA1 gene lacking the region corresponding to Gly61-Gln76 was obtained by digesting the POIA1-encoding plasmid [19] with *Eco*RI and *Pst*I. Then, the synthetic oligonucleotides encoding Gly61-Tyr76 of POIA1cm were ligated with the two fragments described above. Thus constructed plasmid pPOcmSubN encodes the mutated chimera protein of POIA1cm and the N-terminal half of mature subtilisin BPN′.

To construct the expression plasmid of wild-type POIA1-subtilisin BPN′ chimera protein, the plasmid pPOcmSubN was digested with *Pst*I and *Spl*I to remove the region corresponding to the C-terminal region of POIA1cm, and the annealed synthetic oligonucleotides encoding Gly61-Gln76 of the wild-type POIA1 were inserted into this site.

*Corresponding author. Fax: +81 3 5992 1034.
E-mail address: shuichi.kojima@gakushuin.ac.jp (S. Kojima).

Abbreviation: POIA1, *Pleurotus ostrearus* proteinase A inhibitor 1

0014-5793/$30.00
doi:10.1016/j.febslet.2005.06.083

Then, the nucleotide sequence of the *Spl*I site was converted to CCCAGG using Quik Change Mutagenesis Kit to produce a plasmid pPOSubN. To change the processing site -Ala-Gln-Ala↓Ala-Gly- between the signal peptide and the propeptide region of wild-type subtilisin BPN′ to -Ala-Gln-Ala↓Met-Ala-Gly-, the *Nco*I–*Cla*I fragment of pTVSubN encoding the N-terminal half of pro-subtilisin starting with Met [15] was inserted into the *Nco*I–*Cla*I site of pPOcmSubN. Thus constructed plasmid was named pProSubN. Construction of a mutated subtilisin gene which possesses a signal peptide and a mature region but lacks a propeptide region of subtilisin BPN′ was carried out by deletion mutagenesis using Quik Change Mutagenesis Kit using pSUB-N as a template. The partial amino acid sequences around the processing sites of proteins encoded in the plasmids thus constructed are summarized in Fig. 1.

Then, the *Eco*RI–*Hin*dIII fragment of pSUB-C encoding the C-terminal half of subtilisin BPN′ [21] was inserted into the *Eco*RI–*Hin*dIII site of pTZ19U, and the substitution of Ser221 with Cys was carried out by Kunkel's method [22]. A mutated subtilisin BPN′ gene was obtained by digesting a mutated plasmid with *Cla*I and *Bgl*II, and inserted into the *Nco*I–*Bam*HI site of pET11d, along with the *Nco*I–*Cla*I fragment encoding wild-type-like pro-subtilisin BPN′ or the chimera protein in which wild-type or mutated POIA1 was fused to the N-terminus of subtilisin BPN′ to construct an expression plasmid in *E. coli*.

2.2. Expression in E. coli and purification of pro-subtilisin or chimera protein

The expression plasmid of pro-subtilisin or chimera protein with a Ser221 → Cys substitution in subtilisin BPN′ constructed in pET11d was transferred into *E. coli* BL21(DE3). The subsequent cultivation of *E. coli*, and the expression and purification of either protein under denaturing conditions were carried out essentially as described previously [15] with modification, that is, the chimera proteins of POIA1 with subtilisin were purified using DEAE-cellulose.

2.3. In vitro refolding of pro-subtilisin or chimera protein of POIA1 and subtilisin

The concentration of purified pro-subtilisin or the chimera proteins in 6 M urea/50 mM Tris–HCl (pH 7.0) was determined from their absorbance at 280 nm with molar coefficient constants of 34990 $M^{-1}\,cm^{-1}$ for pro-subtilisin, 32490 $M^{-1}\,cm^{-1}$ for POIA1cm-subtilisin and its Phe → Ala mutants and 31240 $M^{-1}\,cm^{-1}$ for POIA1-subtilisin [4,23], and then adjusted to 10 μM.

The protein solutions thus obtained were dialyzed against 50 mM Tris–HCl/0.5 M $(NH_4)_2SO_4$/1 mM $CaCl_2$/5 mM 2-mercaptoethanol (pH 7.0) at 4 °C for 2 days. After the dialysis, proteins were precipitated by adding trichloroacetic acid to a final concentration of 20% and subjected to SDS–polyacrylamide gel electrophoresis.

2.4. Expression of subtilisin BPN′ or chimera protein gene in Bacillus subtilis

A fragment encoding the promoter region and signal peptide of subtilisin BPN′ obtained by amplifying the subtilisin BPN′ gene through PCR was digested with *Dra*I and *Nco*I and inserted into the *Sma*I–*Cla*I site of pSUB-N along with the *Nco*I–*Cla*I fragment obtained by digesting the plasmid pPOSubN, pPOcmSubN or pProSubN. Then, the chimera gene thus constructed was digested with *Eco*RI and *Cla*I, and inserted into the *Eco*RI–*Hin*dIII site of the *E. coli–B. subtilis* shuttle vector pHY300PLK [24] along with the *Cla*I–*Hin*dIII fragment encoding the C-terminal half of subtilisin BPN′ to obtain the expression plasmid of the chimera protein in *B. subtilis*. Then, the expression plasmid was converted into multimerized form by the $recA^+$ strain of *E. coli*, and then introduced into *B. subtilis* UOT0999 using tetracyclin as a selection marker. Transformants were transferred onto an LB plate containing 1% skim milk and incubated at 37 °C for 39 h. For protein analysis, the transformants were inoculated and incubated in 50 ml of LB medium at 37 °C for 2 days, and proteins in the culture supernatants were precipitated by adding trichloroacetic acid to a final concentration of 20% and subjected to SDS–polyacrylamide gel electrophoresis.

2.5. Measurements of proteolytic activity

Proteolytic activity in the supernatants of 50 ml culture of *Bacillus* cells incubated for 2 days was measured at 25 °C using 0.1 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide in 0.1 M Tris–HCl (pH 8.6) by monitoring increase of the absorbance at 410 nm.

2.6. Site-directed mutagenesis

The substitution of Phe at position 44 or 56 of POIA1cm in the chimera protein with subtilisin BPN′ was carried out using the Quik Change Mutagenesis Kit with pPOcmSubN as a template and mutation primers designed to replace the Phe codon (TTC) with the Ala codon (GCC). The expression plasmid in *E. coli* or *B. subtilis* from pPOcmSubN was constructed as described above.

3. Results

3.1. In vitro experiments: subtilisin refolding and processing by chimera proteins

To determine the possibility of POIA1 functioning as an intramolecular chaperone of subtilisin BPN′ in vitro, wild-type or mutated POIA1 (POIA1cm) whose C-terminal six residues had been substituted with those of the propeptide of subtilisin BPN′ was fused to the N-terminus of mature subtilisin BPN′ in which catalytic Ser221 was substituted with Cys, and the chimera proteins thus constructed were expressed in *E. coli*. Since pro-subtilisin BPN′ with Cys as a catalytic residue has been shown to be specifically cleaved at only the junction site between the propeptide and mature regions without resulting in the subsequent autodegradation of the propeptide [4], the POIA1-mediated refolding of subtilisin BPN′ was investigated on the basis of the occurrence of this processing, making the conclusion more simple than the case in which autodegradation of subtilisin BPN′ would occur by the presence of catalytic Ser221. Because the mutational analyses of the propeptide of subtilisin BPN′ have shown that its intramolecular chaperone activity is closely related to its ability to bind to a cognate protease [10–15] and our previous study has demonstrated that the substitution of C-terminal six residues with those of the

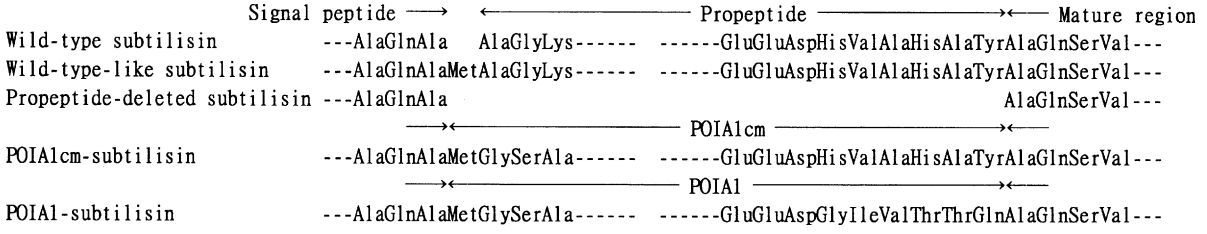

Fig. 1. Junction sequences between signal peptide, propeptide or POIA1 and mature region of subtilisin BPN′ of wild-type-like subtilisin BPN′ or fused protein of POIA1 and subtilisin BPN′, and that between signal peptide and mature region of subtilisin BPN′ of propeptide-deleted subtilisin BPN′ constructed in this study. To make the junction sequence uniform, the sequence between the signal peptide and propeptide of wild-type subtilisin BPN′ was changed to produce wild-type-like subtilisin BPN′.

propeptide markedly enhances the affinity of POIA1 to subtilisin BPN′ [19], POIA1cm is expected to exhibit a stronger activity than the wild-type POIA1, when both function as intramolecular chaperones.

The chimera protein in which wild-type POIA1 or POIA1cm was fused to the N-terminus of subtilisin BPN′ with a Ser221 → Cys substitution was shown to be expressed as an inclusion body in *E. coli*. They were purified in the presence of 6 M urea after the solubilization of the inclusion bodies in 6 M GdnHCl and dialysis. Pro-subtilisin BPN′ with a Ser221 → Cys substitution was also purified in a similar way. The purified proteins were adjusted to a concentration of 10 μM and then subjected to dialysis against a refolding buffer containing 0.5 M $(NH_4)_2SO_4$, 1 mM $CaCl_2$ and 5 mM 2-mercaptoethanol. After dialysis at 4 °C for 2 days, proteins in the dialysis apparatus were precipitated with trichloroacetic acid and analyzed by SDS–PAGE.

As shown in Fig. 2, all the samples produce two bands that correspond to processed species (subtilisin BPN′ and wild-type POIA1, POIA1cm or propeptide) in addition to unprocessed species, indicating that wild-type POIA1 and POIA1cm can function as intramolecular chaperones of subtilisin BPN′, in a similar way to the propeptide, although their activities differs in their extent. Among the samples, the difference in the total amount of protein on the gel, in spite of the same amount loaded, was considered to be related to the differences in the amount of precipitate during dialysis. It was found that pro-subtilisin hardly produced precipitates, but that approximately one-half the amount of fused proteins with wild-type POIA1 or POIA1cm was precipitated during dialysis. Electrophoretic analysis showed that the precipitates were mainly composed of an unprocessed fused protein and subtilisin BPN′, and that their amount ratio was similar to that in soluble fractions (data not shown). Since the precipitates formed during dialysis are considered to be the results of misfolding, the amount of processed subtilisin BPN′ molecules after dialysis seems to be related to the intramolecular chaperone activity of the propeptide or POIA1 in vitro.

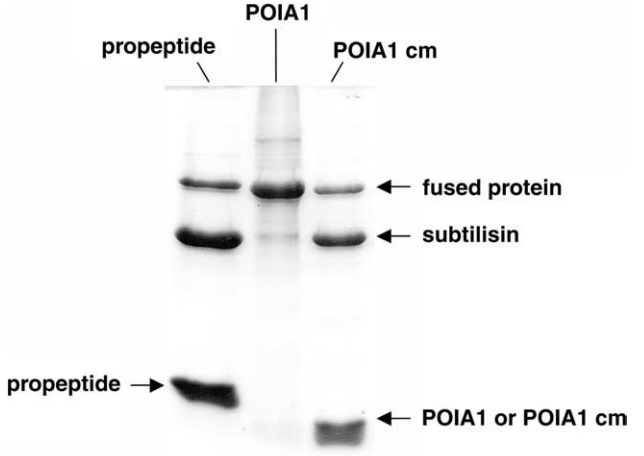

Fig. 2. In vitro intramolecular chaperone activity of propeptide, wild-type POIA1 or POIA1cm on the basis of occurrence of processing in pro-subtilisin or chimera protein after refolding. Pro-subtilisin with a Ser221 → Cys substitution or chimera protein in which wild-type POIA1 or POIA1cm was attached to the N-terminus of subtilisin BPN′ with a Ser221 → Cys substitution was purified in the presence of 6 M urea and its concentration was adjusted to 10 μM. The protein solution thus obtained was subjected to dialysis against 50 mM Tris–HCl/0.5 M $(NH_4)_2SO_4$/1 mM $CaCl_2$/5 mM 2-mercaptoethanol (pH 7.0) at 4 °C for 2 days. Proteins in an aliquot of 40 μl were precipitated by adding trichloroacetic acid to a final concentration of 20% and analyzed by SDS–polyacrylamide gel electrophoresis.

The electrophoretic pattern in Fig. 2 shows that the amount of subtilisin BPN′ from pro-subtilisin was most abundant and that the amount of subtilisin BPN′ from the chimera protein with wild-type POIA1 was considerably lower than other two cases, indicating that wild-type POIA1 is a much less effective intramolecular chaperone than POIA1cm or the propeptide, as expected. The fact that the chimera protein with POIA1cm produced the aggregates after dialysis suggests that POIA1cm is a less effective intramolecular chaperone than the propeptide, although the amount ratios of subtilisin BPN′ to unprocessed species in the soluble fraction are similar for the propeptide and POIA1cm.

3.2. *In vivo experiments: subtilisin production from chimera gene of POIA1 and subtilisin BPN′*

In the in vitro experiments described above, the function of POIA1 as an intramolecular chaperone was demonstrated on the basis of the occurrence of autoprocessing in the chimera protein of POIA1 and subtilisin BPN′. We also examined the function of POIA1 as an intramolecular chaperone in vivo. The propeptide-encoding region of the subtilisin BPN′ gene was substituted with the wild-type POIA1 or POIA1cm gene, and the chimera gene thus constructed was inserted into a shuttle vector of *E. coli* and *B. subtilis*. After the transformation of *B. subtilis*, the secretory production of active and free subtilisin BPN′ molecules was examined on the basis of the formation of halos on the skim milk plate. The original cleavage sequence between the signal peptide and the propeptide of subtilisin BPN′ was estimated to be Ala-Ala [25,26], whereas the corresponding sequence of our constructs, Ala-Met. Since cleavage by a signal peptidase was considered to depend on the amino acid at the N-terminal side of the cleavage site, it seemed that the effect of our sequence on the secretory production of active subtilisin BPN′ was negligible.

As shown in Fig. 3A, a halo was formed around a *Bacillus* colony transformed with a plasmid encoding a chimera gene of subtilisin BPN′ and wild-type POIA1 or POIA1cm. This result strongly suggests that both wild-type POIA1 and POIA1cm can function as intramolecular chaperones necessary for the correct folding of subtilisin BPN′ to produce an active species. As a control, when a plasmid encoding the signal peptide and the mature region of subtilisin BPN′ but lacking the propeptide region was introduced into *Bacillus* cells, a halo was not observed around its colony. In contrast, a *Bacillus* colony transformed with a plasmid encoding the wild-type-like subtilisin BPN′ gene was found to form a halo of a much larger diameter than that in the case of a *Bacillus* colony transformed with a chimera gene of POIA1 and subtilisin BPN′, despite the fact that in this construct of wild-type-like subtilisin BPN′, the junction sequence between the signal peptide and the propeptide was changed from Ala-Ala to Ala-Met to introduce the same junction sequence of the chimera gene of POIA1cm and subtilisin BPN′. Relative proteolytic activities in the culture supernatants after 2 day-cultivation, which were measured as rates of increasing absorbance at 410 nm by release of *p*-nitroaniline from a synthetic substrate, were 25, 1 and 0.27 for the *Bacillus* cells transformed with a plasmid

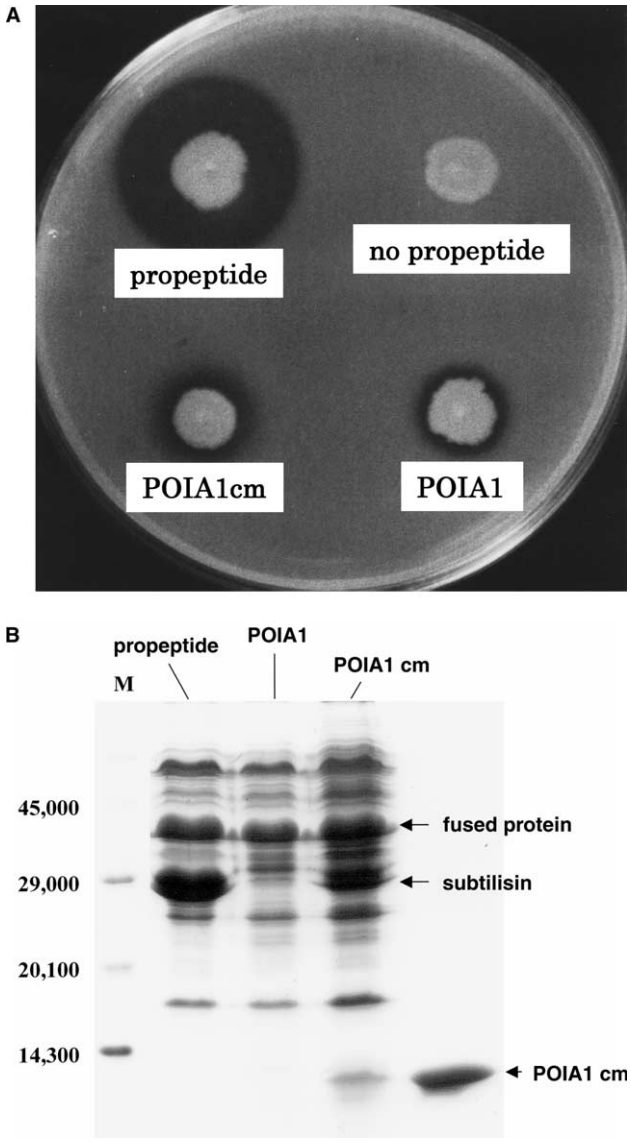

Fig. 3. (A) In vivo intramolecular chaperone activity of propeptide, wild-type POIA1 or POIA1cm detected on the basis of halo formation around *Bacillus* cells. *B. subtilis* UOT0999 cells were transformed with multimerized forms of the plasmid encoding the wild-type-like subtilisin BPN′ gene or chimera gene in which the propeptide region was substituted with wild-type POIA1 or POIA1cm, and inoculated onto an LB plate containing 1% skim milk and tetracyclin, and incubated at 37 °C for 39 h. (B) Electrophoretic analysis of proteins in the culture supernatants of *Bacillus* cells transformed with a plasmid encoding the propeptide, wild-type POIA1 or POIA1cm. After 2 day-cultivation at 37 °C, proteins in a 1 ml aliquot of 50 ml culture were precipitated with trichloroacetic acid, and subjected to SDS–polyacrylamide gel electrophoresis. M, molecular weight markers.

encoding the wild-type-like subtilisin BPN′, chimera protein with POIA1cm and chimera protein with wild-type POIA1, respectively.

The in vitro experiments indicated that POIA1cm is a much more effective intramolecular chaperone than wild-type POIA1. However, a halo around a *Bacillus* colony transformed with a plasmid encoding the chimera protein with POIA1cm is slightly larger than those with a plasmid encoding the chimera protein with wild-type POIA1. Such difference in halo diameter was considered to be due to the difference in the number of active subtilisin molecule. We previously demonstrated that POIA1cm is a strong and resistant inhibitor of subtilisin BPN′, whereas wild-type POIA1 is a digestible inhibitor. Therefore, the reason for the *Bacillus* colony transformed with a plasmid with the POIA1cm gene forming a slightly larger halo than that transformed with wild-type POIA1 gene was thought to be the marked reduction in the number of active and free subtilisin BPN′ molecules after processing caused by the tight binding of subtilisin BPN′ to POIA1cm in spite of POIA1cm being sufficiently active to function as an intramolecular chaperone.

With the above considerations, we also have carried out electrophoretic analysis of proteins in the culture supernatants of *Bacillus* cells cultivated for 2 days. As shown in Fig. 3B, the *Bacillus* cells transformed with a plasmid encoding the wild-type-like subtilisin BPN′ gene produced a large amount of subtilisin molecule, although a comparable amount of pro-subtilisin molecule was also observed. The band of the propeptide was not detected as expected, due to its degradation by active subtilisin molecules. In the culture supernatants of the *Bacillus* cells transformed with a plasmid encoding POIA1cm, a band corresponding to POIA1cm was detected in addition to the bands of processed subtilisin BPN′ and unprocessed chimera protein. Therefore, the proteolytic activity of processed subtilisin molecules was largely suppressed by binding with POIA1cm which is a strong and resistant inhibitor of subtilisin BPN′. In contrast, the amount of subtilisin BPN′ in the culture supernatants of the *Bacillus* cells transformed with a plasmid encoding wild-type POIA1 was very low, and the band of POIA1 was hardly detected maybe due to its degradation by subtilisin BPN′, thus leading to the formation of only a small halo on the skim milk plate.

These results by electrophoretic analysis are just consistent with those by the in vitro experiments and inhibitory properties of the propeptide and POIA1, and can well explain the diameter of halos on the skim milk plate and proteolytic activities in the culture supernatants.

3.3. Effects of Phe → Ala substitution at different sites in POIA1 on its intramolecular chaperone activities in vitro and in vivo

The in vitro and in vivo experiments described above clearly show that POIA1 can function as an intramolecular chaperone, although it was originally discovered as a protease inhibitor. In our previous studies on POIA1 as a protease inhibitor, we found that a Phe → Ala substitution at different sites of POIA1cm produces different effects on the POIA1 structure and functions [27]. When Phe at position 56 of POIA1cm was substituted with Ala, the resultant mutant of POIA1 temporarily inhibited subtilisin BPN′, in spite of the retention of its tertiary structure as determined by CD spectral measurements. In contrast, it was found that a Phe → Ala substitution at position 44 results in the loss of the POIA1 tertiary structure and the conversion of POIA1 to a temporary inhibitor. We therefore examined the effects of these substitutions on the intramolecular chaperone activity of POIA1, and investigated its relation to the characteristics as a protease inhibitor and the tertiary structure of POIA1.

Fig. 4 shows that a chimera protein of POIA1cm with a Phe56 → Ala substitution and subtilisin BPN′ produced two additional bands corresponding to processed species whose amounts are comparable to that of POIA1cm; no bands corresponding to processed species are observed for a chimera

protein of POIA1cm with a Phe44 → Ala substitution and subtilisin BPN′. Similarly, Fig. 5A shows that the *Bacillus* colony transformed with a chimera gene encoding POIA1cm with a Phe56 → Ala substitution and subtilisin BPN′ formed a much larger halo on the skim milk plate than that transformed with POIA1cm. In contrast, no halo was formed around the *Bacillus* colony transformed with a chimera gene with a Phe44 → Ala substitution in POIA1cm. Electrophoretic pattern in Fig. 5B also shows that the band of processed subtilisin BPN′ was hardly detected in the culture supernatants of the *Bacillus* cells transformed with a chimera gene encoding POIA1cm with a Phe44 → Ala substitution, whereas the *Bacillus* cells transformed with a chimera gene with a Phe56 → Ala substitution produced the band of active and free subtilisin molecules. Relative proteolytic activities in the culture supernatants measured as rates of increasing absorbance at 410 nm by hydrolysis of a synthetic substrate were 3.6, 1 and 0.04 for the *Bacillus* cells transformed with a plasmid encoding chimera protein with POIA1cm with a Phe56 → Ala substitution, chimera protein with POIA1cm and chimera protein with POIA1cm with a Phe44 → Ala substitution, respectively.

These results clearly demonstrate that the intramolecular activity of POIA1 is closely related to the tertiary structure and inhibitory properties of POIA1. The Phe44 → Ala mutant of POIA1cm lacked an intramolecular chaperone activity; this might have been due to the loss of its tertiary structure caused by the substitution. The Phe56 → Ala mutant of POIA1cm retained its tertiary structure, as in the case of POIA1cm; this might have led to its in vitro intramolecular chaperone activity being similar to that of POIA1cm. Since a Phe56 → Ala substitution converts POIA1cm from a resistant inhibitor into a digestible temporary inhibitor, the POIA1cm portion with a Phe56 → Ala substitution of a secreted chimera protein is degraded by an active species of subtilisin BPN′ more easily than POIA1cm, thus producing a larger halo on the skim milk plate. Thus, the Phe56 → Ala mutant of POIA1cm that possesses a tertiary structure but has been converted into a digestible temporary inhibitor is the most effective intramolecular chaperone

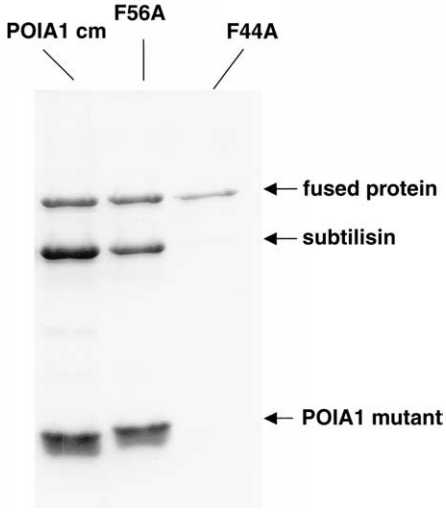

Fig. 4. In vitro intramolecular chaperone activity of POIA1cm, or its Phe44 → Ala or Phe56 → Ala mutant on the basis of occurrence of processing in chimera protein after refolding. Details are essentially the same as those described in the legend of Fig. 2.

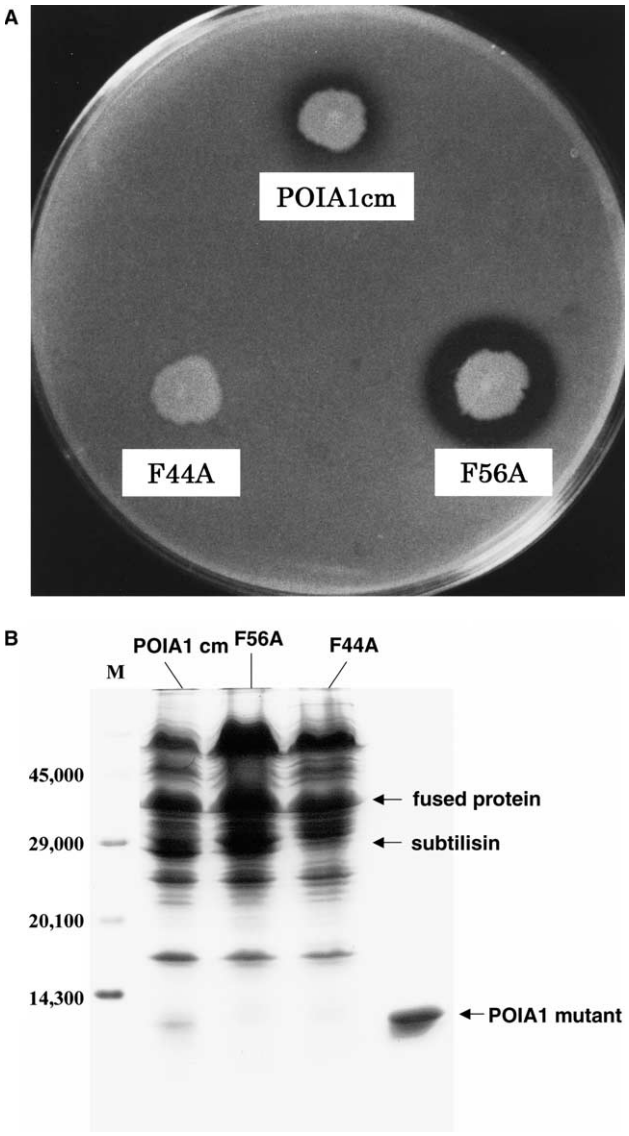

Fig. 5. (A) In vivo intramolecular chaperone activity of POIA1cm, or its Phe44 → Ala or Phe56 → Ala mutant on the basis of halo formation around *Bacillus* cells. (B) Electrophoretic analysis of proteins in the culture supernatants of *Bacillus* cells transformed with a plasmid encoding POIA1cm, or its Phe44 → Ala or Phe56 → Ala mutant. Details are essentially the same as those described in the legend of Fig. 3. M, molecular weight markers.

to form a large halo among the POIA1 mutants examined in this study, although its activity is still weaker than that of the propeptide of subtilisin BPN′.

4. Discussion

Although POIA1 had been isolated as an endogenous inhibitor of *P. ostreatus* proteinase A [17], the fact that its amino acid sequence is homologous to those of the propeptides of subtilisin by about 30% prompted us to examine the possible functional similarity between these proteins. In our previous studies on POIA1, we have demonstrated that POIA1 is unique among serine protease inhibitors, in that it utilizes its C-terminal region for binding to and inhibiting subtilisin BPN′

[19], as in the case of the propeptide of subtilisin BPN′ [7–9]. Therefore, POIA1, which is a digestible temporary inhibitor of subtilisin BPN′, could be converted into a resistant and strong inhibitor of subtilisin BPN′ by substituting its C-terminal six residues with those of the propeptide of subtilisin BPN′. In this study, we have shown that POIA1 can function as an intramolecular chaperone of subtilisin BPN′ in vitro and in vivo using chimera genes with subtilisin BPN′, in which the propeptide-encoding region has been substituted with the POIA1 gene.

In vitro experiments have shown that POIA1cm is a much more effective intramolecular chaperone of subtilisin BPN′ than wild-type POIA1, suggesting that the tight binding of POIA1cm with subtilisin BPN′ as a result of the substitution of its C-terminal six residues with those of the propeptide of subtilisin BPN′ is necessary for the effective intramolecular chaperone activity of POIA1. A similar close relationship between affinity to subtilisin BPN′ and intramolecular chaperone activity for the propeptide of subtilisin BPN′ was also demonstrated by Bryan et al., although they have analyzed C-terminal truncated mutants of the propeptide [28]. However, the number of active subtilisin BPN′ molecules determined from the diameter of the halo formed on the skim milk plate as in vivo intramolecular chaperone activity of POIA1cm was found to be only slightly higher than that of the wild-type POIA1 and much lower than that of the propeptide, apparently. These differences between the results of in vitro and in vivo experiments were considered to be due to the differences between the inhibitory properties of these proteins. Since POIA1cm is a resistant and strong inhibitor of subtilisin BPN′ [19], its degradation by subtilisin BPN′ proceeds very slowly compared with that of wild-type POIA1 and the propeptide, as shown by the electrophoretic analysis of proteins in the culture supernatants of the *Bacillus* cells, thus leading to the release of only a small number of active and free subtilisin BPN′ molecules. In contrast, the propeptide of subtilisin BPN′ is a temporary inhibitor of subtilisin BPN′ that is more rapidly degraded by subtilisin BPN′ [3,29] than POIA1cm, thus resulting in the formation of a large halo. Wild-type POIA1, which is also a digestible temporary inhibitor of subtilisin BPN′, was found to be a much less effective intramolecular chaperone of subtilisin BPN′ as determined in the same in vitro experiments described above. Therefore, the *Bacillus* cell transformed with a chimera gene of wild-type POIA1 and subtilisin BPN′ showed the smallest halo among the cells transformed with three genes.

Then, a question arises whether the tight binding to subtilisin BPN′ is sufficient for the intramolecular chaperone activity of POIA1. We previously demonstrated that a Phe → Ala substitution at different sites of POIA1cm converts POIA1cm into a digestible temporary inhibitor, but produces different effects on the POIA1 tertiary structure [27]. The Phe56 → Ala substitution in POIA1cm resulted in the maintenance of POIA1 tertiary structure, whereas the Phe44 → Ala substitution resulted in the loss of such a tertiary structure, as determined by CD spectral measurements. Therefore, we have examined the relationship between the tertiary structure and intramolecular chaperone activity of POIA1. As shown by in vitro and in vivo experiments, POIA1cm with a Phe44 → Ala substitution could not function as an intramolecular chaperone of subtilisin BPN′, because processed species or halos on the skim milk plate were hardly formed from the chimera protein and around a *Bacillus* colony, respectively. Upon this substitution, the ability of POIA1cm to bind to subtilisin BPN′ was weakened by about 80-fold, and this decrease in the extent of interaction was also considered to be the reason for the loss of the intramolecular chaperone activity of the Phe44 → Ala mutant of POIA1cm. However, the amino acid sequence of the POIA1 C-terminal region, which is the main region responsible for binding to subtilisin BPN′, was the same as that of POIA1cm, and the decrease in the extent of the interaction of this mutant with subtilisin BPN′ was thought to be due to the entropic effects caused by the loss of the POIA1 tertiary structure. Therefore, we considered that the loss of the intramolecular chaperone activity of the Phe44 → Ala mutant of POIA1cm is mainly due to the loss of its tertiary structure.

In a parallel study, we have determined the solution structure of POIA1 by NMR spectroscopy, and found that the POIA1 tertiary structure is very similar to that of the propeptide of subtilisin BPN′ in complex with a cognate protease [20], in spite of a sequence homology of only approximately 30%. Therefore, such a POIA1 tertiary structure seems to be an inevitable requirement for the intramolecular chaperone activity of POIA1. This situation in POIA1, however, is somewhat different from that in the propeptide. The propeptide of subtilisin BPN′ does not have a defined tertiary structure in isolated form, but it acquires a tertiary structure upon the formation of a complex with a cognate protease [4–6]. Although the detailed molecular mechanisms of such a phenomenon in the propeptide have not been clarified yet, it is considered that similar phenomena do not occur in the Phe44 → Ala mutant of POIA1cm, since this mutant could not function as an intramolecular chaperone. However, our groups and others have demonstrated that the formation of the tertiary structure of a propeptide by mutation introduction has produced positive effects on the intramolecular chaperone activity of the propeptide [10–15].

A comparison of the propeptide of subtilisin BPN′, wild-type POIA1, POIA1cm and its Phe44 → Ala mutant has demonstrated that the tertiary structure of POIA1 which is similar to that of the propeptide of subtilisin BPN′, is necessary for the function of POIA1 as an intramolecular chaperone, and that an increased ability to bind to subtilisin BPN′ makes POIA1 a more effective intramolecular chaperone of subtilisin BPN′. However, since POIA1cm is a resistant inhibitor of subtilisin BPN′, it could not be degraded by subtilisin BPN′ after functioning as an intramolecluar chaperone, thus leading to the formation of a halo smaller than that obtained in the case of the propeptide of subtilisin BPN′. This is in marked contrast to the case of the propeptide. Therefore, if a POIA1 mutant that is digestible by subtilisin BPN′ and retains its tertiary structure could be generated, such a mutant would function as an effective intramolecular chaperone, which could produce active and free subtilisin BPN′ molecules. In our previous study on POIA1, we have identified a Phe56 → Ala substitution in POIA1 to be suitable for satisfying this criterion. Fig. 4A shows that the *Bacillus* colony transformed with the chimera gene in which a Phe56 → Ala substitution was introduced into the POIA1cm region exhibits a halo larger than that in the case of the *Bacillus* colony transformed with the POIA1cm gene.

In conclusion, we have demonstrated that POIA1, which had been originally discovered as a protease inhibitor with a sequence homology of only approximately 30% to the propeptide

of subtilisin BPN′, can function as an intramolecular chaperone of subtilisin BPN′, and that its activity is closely related to its tertiary structure, which resembles that of the propeptide in complex with subtilisin BPN′, and its ability to bind to a protease. We are currently investigating in more detail the requirements of POIA1 for its function as an intramolecular chaperone by analyzing other POIA1 mutants. Through such studies, the molecular mechanisms of the propeptide-mediated refolding of subtilisin BPN′ will be clarified from different viewpoints. In addition, some hints on the evolutional origin of the propeptide may be provided in the future.

Acknowledgement: This work was supported by a grant from the Naito Foundation.

References

[1] Shinde, U. and Inouye, M. (1993) Intramolecular chaperones and protein folding. Trends Biol. Sci. 18, 442–447.
[2] Ohta, Y. and Inouye, M. (1990) Pro-subtilisin E: Purification and characterization of its autoprocessing to active subtilisin E in vivo. Mol. Microbiol. 4, 295–304.
[3] Hu, Z., Haghjoo, K. and Jordan, F. (1996) Further evidence for the structure of the subtilisin propeptide and for its interactions with mature subtilisin. J. Biol. Chem. 271, 3375–3384.
[4] Eder, J., Rheinnecker, M. and Fersht, A.R. (1993) Folding of subtilisin BPN′: Role of the pro-sequence. J. Mol. Biol. 233, 293–304.
[5] Strausberg, S., Alexander, P., Wang, L., Schwarz, F. and Bryan, P. (1993) Catalysis of a protein folding reaction: Thermodynamic and kinetic analysis of subtilisin BPN′ interactions with its propeptide fragment. Biochemistry 32, 8112–8119.
[6] Shinde, U., Li, Y., Chatterjee, S. and Inouye, M. (1993) Folding pathway mediated by an intramolecular chaperone. Proc. Natl. Acad. Sci. USA 90, 6924–6928.
[7] Gallagher, T., Gilliland, G., Wang, L. and Bryan, P. (1995) The prosegment-subtilisin BPN′ complex: Crystal structure of a specific foldase. Structure 3, 907–914.
[8] Bryan, P., Wang, L., Hoskins, J., Ruvinov, S., Strausberg, S., Alexander, P., Almog, O., Gilliland, G. and Gallagher, T. (1995) Catalysis of a protein folding reaction: Mechanistic implications of the 2.0 Å structure of the subtilisin-prodomain complex. Biochemistry 34, 10310–10318.
[9] Janin, S.C., Shinde, U., Li, Y., Inouye, M. and Berman, H. (1998) The crystal structure of an autoprocessed Ser221Cys-subtilisin E-propeptide complex at 2.0 Å resolution. J. Mol. Biol. 284, 137–144.
[10] Ruvinov, S., Wang, L., Ruan, B., Almog, O., Gilliland, G.L., Einstein, E. and Bryan, P.N. (1997) Engineering the independent folding of the subtilisin BPN′ prodomain: Analysis of two-state folding versus protein stability. Biochemistry 36, 10414–10421.
[11] Ruan, B., Hoskins, J., Wang, L. and Bryan, P.N. (1998) Stabilizing the subtilisin BPN′ pro-domain by phage display selection: How restrictive is the amino acid code for maximum protein stability? Protein Sci. 7, 2345–2353.
[12] Kojima, S., Minagawa, T. and Miura, K. (1998) Tertiary structure formation in the propeptide of subtilisin BPN′ by successive amino acid replacements and its close relation to function. J. Mol. Biol. 277, 1007–1013.
[13] Wang, L., Ruan, B., Ruvinov, S. and Bryan, P.N. (1998) Engineering the independent folding of the subtilisin BPN′ pro-domain: Correlation of pro-domain stability with the rate of subtilisin folding. Biochemistry 37, 3165–3171.
[14] Ruan, B., Hoskins, J. and Bryan, P.N. (1999) Rapid folding of calcium-free subtilisin by a stabilized pro-domain mutant. Biochemistry 38, 8562–8571.
[15] Kojima, S., Yanai, H. and Miura, K. (2001) Accelerated refolding of subtilisin BPN′ by tertiary-structure-forming mutants of its propeptide. J. Biochem. 130, 471–474.
[16] Maier, K., Muller, H., Tesch, R., Trolp, R., Witt, I. and Holzer, H. (1979) Primary structure of yeast proteinase B inhibitor 2. J. Biol. Chem. 254, 12555–12561.
[17] Dohmae, N., Takio, K., Tsumuraya, Y. and Hashimoto, Y. (1995) The complete amino acid sequences of two serine proteinase inhibitors from the fruiting bodies of a basidiomycetes, *Pleurotus ostreatus*. Arch. Biochem. Biophys. 316, 498–506.
[18] Kojima, S., Deguchi, M. and Miura, K. (1999) Involvement of the C-terminal region of yeast proteinase B inhibitor 2 in its inhibitory action. J. Mol. Biol. 286, 775–785.
[19] Kojima, S., Hisano, Y. and Miura, K. (2001) Alteration of inhibitory properties of *Pleurous ostreatus* proteinase A inhibitor 1 by mutation of its C-terminal region. Biochem. Biophys. Res. Commun. 281, 1271–1276.
[20] Sasakawa, H., Yoshinaga, Kojima, S. and Tamura, A. (2002) Structure of POIA1, a homologous protein to the propeptide of subtilisin: Implication for protein foldability and the function as an intramolecular chaperone. J. Mol. Biol. 317, 159–167.
[21] Masuda-Momma, K., Shimakawa, T., Inouye, K., Hiromi, K., Kojima, S., Kumagai, I., Miura, K. and Tonomura, B. (1993) Identification of amino acid residues responsible for the changes of absorption and fluorescence spectra on the binding of subtilisin BPN′ and *Streptomyces* subtilisin inhibitor. J. Biochem. 114, 906–911.
[22] Kunkel, T.A. (1985) Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82, 488–492.
[23] Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182, 319–326.
[24] Ishiwa, H. and Shibahara, H. (1985) New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. II. Plasmid pHY300PLK, a multipurpose cloning vector with a polylinker, derived from pHY460. Jpn. J. Genet. 60, 235–243.
[25] Wells, J.A., Ferrari, M., Henner, D.J., Estell, D.A. and Chen, E.Y. (1983) Cloning, sequencing and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus* subtilis. Nucleic Acid Res. 11, 7911–7924.
[26] Vasantha, N., Thompson, L.D., Rhides, C., Banner, C., Nagle, J. and Filpula, D. (1984) Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. J. Bacteriol. 159, 811–819.
[27] Kojima, S. and Hisano, Y. (2002) Requirement for hydrophobic Phe residues in *Pleurotus ostreatus* proteinase A inhibitor 1 for stable inhibition. Protein Eng. 15, 325–329.
[28] Wang, L., Ruvinov, S., Strausberg, S., Gallagher, T., Gilliland, G. and Bryan, P.N. (1995) Prodomain mutations at the subtilisin interface: Correlation of binding energy and the rate of catalyzed folding. Biochemistry 34, 15415–15420.
[29] Kojima, S., Minagawa, T. and Miura, K. (1997) The propeptide of subtilisin BPN′ as a temporary inhibitor and effect of an amino acid replacement on its inhibitory activity. FEBS Lett. 411, 128–132.